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Title: Salvinorin A content in legal high products of *Salvia divinorum* sold in Mexico

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Salvinorin A content in legal high products of *Salvia divinorum* sold in Mexico.

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Salvinorin A content in legal high products of *Salvia divinorum* sold in Mexico.

Abstract

*Salvia divinorum* (Lamiaceae) is a herb native to Mexico where it is used by Mazatec shamans for spiritual and divination purposes. *S. divinorum* products are easily available to consumers and are used worldwide as legal highs because of the hallucinogenic effects caused mainly by salvinorin A. Highly popular videos and websites in the internet depicting the use of *S. divinorum* products have contributed to an increase in their consumption. Recent reports have highlighted the potential of these products to induce psychosis in consumers. In Mexico, dried leaf extracts of *S. divinorum* are sold in different strengths, claiming to correlate with increasing amounts of salvinorin A. In order to determine the variability of salvinorin A content between brands and to investigate possible correlation between brand strengths, this study sought to quantify salvinorin A in commercial products available in Mexico using an HPLC method. The HPLC analytical method showed a correlation coefficient $R^2>0.99$, with LOD of 0.44 $\mu$g/mL and LOQ of 1.34 $\mu$g/mL. The retention time for salvinorin A was 23.09 ± 0.95 min and the measured concentrations ranged between 8.32 ± 0.65 to 56.52 ± 3.77 mg/g dried leaf. The results for brand c did not show an agreement between the declared and the calculated amount of salvinorin A. Additionally, the emergence in Mexico of high strength salvia products (100x), the lack of regulation and the observed variability of salvinorin A content
between brands of commercial legal highs products of *S. divinorum* could result in a health problem for consumers.

**Keywords**


1. Introduction

In recent years, the use of a group of substances legally sold and intended to induce behavioural effects similar to illegal substances, known as legal highs, has been popularized [1]. These new psychoactive substances, are also known as designer drugs, herbal highs or research chemicals and they can be of synthetic or natural origin [2,3]. Additionally, there is a third category of legal highs known as “spices” consisting of mixtures of synthetic cannabinoids with plants [4]. Several plant-based legal highs are available in Mexico, these include the dream herb (*Calea zacatechichi*), kratom (*Myrtogina speciosa*), blue lotus (*Nimphae caeruluae*) and ska Maria pastora (*Salvia divinorum*). *Salvia divinorum* Epling and Játiva-M (Lamiaceae) is a shrub endemic in Oaxaca, Mexico. It has been used by Mazatec shamans as part of spiritual and divination rituals because of its well documented hallucinogenic effects. However, its main current use is as a legal high [5,6]. Originally, in order to induce these hallucinogenic effects, an infusion of 20-80 pairs of fresh leaves of *S. divinorum* was typically prepared. However, it is currently a common practice to smoke the dried leaves (4-5 pairs), although they can also be chewed fresh [7]. *S. divinorum* is also used for medicinal purposes as anti-
parasitic, anti-diarrhoea and a cure for an abdominal inflammation condition called "panzón de borrego" [8, 9].

Previous phytochemical studies of *S. divinorum* have led to the isolation of several diterpenes including salvinorins A-J, saldividins A-D, divinatorins D and F, and salvinicins A and B [7, 10, 11]. Salvinorin A (Figure 1), the main active diterpene of this plant has shown diverse biological effects such as anti-inflammatory [12], antidepressant [13], gastrointestinal [14] and hallucinogenic [15]. Previous *in vitro* and *in vivo* pharmacological studies have established salvinorin A as an allosteric kappa opioid receptor (KOR) agonist [16]. Contrary to other commonly used hallucinogens, salvinorin A has not been reported to have agonistic effects on 5-HT, CB1, CB2, NMDA and muscarinic receptors [17].
Because of its current popular use as a legal high, *S. divinorum* is sold as tinctures or dry-leaf extracts in the internet and in stores called "smart shops" [1]. These products are sold at different “strengths”, which supposedly correlate with the concentration of salvinorin A in the product [10, 18]. The U.S. and some European countries such as Belgium, Denmark, Italy and Spain have already established legal standards for selling or preventing the sale of *S. divinorum* products; however countries like Mexico currently lack such regulations [19]. The Mexican Government has publicly expressed a desire to regulate the sales and use of this plant by including *S. divinorum* and salvinorin A in the list of psychotropic
substances of the “Ley General de Salud” (Mexican Health and Safety Law) [20]. In countries other than Mexico, the consumption of *S. divinorum*, has been associated with one case of schizophrenia [21], psychotimetic effects [22], and gastrointestinal disorders [23]. In 2013, this plant was listed in the World Drug Report by the United Nations Office on Drugs and Crime as one of the new psychoactive substances (NPS) to be monitored as it could represent a public health threat [24].

The aim of this investigation was to establish and validate an HPLC method to quantify the content of salvinorin A in *S. divinorum* products sold in Mexico, in order to determine the variability in the content of the bioactive diterpene in different *S. divinorum* products and assess the variability in their content of salvinorin A between products of different brands with similar strength.
2. Material and methods

2.1 Samples and chemicals.

Twelve commercial products belonging to three commercial brands were legally purchased via the internet from Mexican internet retailers. Eleven of the products were labelled as concentrated dried leaf extracts, all identified as *Salvia divinorum*. The products were labelled as a variety of “strengths” from 5X to 100X. The reference standard salvinorin A (purity ≥ 98%) was purchased from Sigma-Aldrich (Mexico). HPLC grade solvents methanol and acetonitrile, and formic acid were obtained from JT Baker (USA). The deionized water used was of Milli-Q quality.

2.2 Standard solutions.

A 1 mg/mL stock solution of salvinorin A was prepared in a methanol-water (9:1) mixture. Serial working dilutions (200 to 3.125 µg/mL) were prepared from the stock solution using methanol-water (9:1).

2.3 Extraction of the samples

An aliquot of the dried leaf product was placed in a mortar and pulverized to a fine powder (particle size: 5 µm). For the extraction, a standardized method using HPLC grade solvents was used [25]. Briefly, 10 mg of product were placed in a 1.5 mL Eppendorf tube and 1 mL of a methanol-water mixture (9:1) was added. The tubes were sonicated for 1 h in a water bath at 35 °C, then centrifuged (Eppendorf 5417C) at 3342.85 x g (3000 r.p.m) at room temperature (23 °C/air-conditioned) for
30 minutes. An aliquot of 100 μL of the supernatant was recovered using a micropipette and diluted 1:10 with a mixture of acetonitrile-water (9:1). The samples were analysed on three different days in triplicate; a mixture of 0.01% formic acid-acetonitrile was used as a blank.

2.4 Chromatographic conditions.

Salvinorin A concentrations were quantified using a Waters 600 C HPLC system equipped with an auto sampler (Waters 717 Plus). The chromatographic separation was performed with an Eclipse XDB-C18 column (150 mm x 4.6 mm id, 5 μm Agilent, UK) at room temperature (23 °C/air-conditioned). The injection volume was 20 μL and the flow rate was kept at 1 mL/min. The mobile phase consisted of a solution of 0.01% formic acid (A) and acetonitrile (B). The gradient elution was performed as follows; 0-1 min 25% (B); 1-30 min gradient 90% (B); 30-35 min 10% (B); 35-40 min gradient 25% (B) and 40-45 min 25% (B). Sample injections were performed in triplicate, and data recorded using an absorbance detector (Waters 2487) set at 210 nm.

2.5 Quantitative Analysis

Calibration curves of salvinorin A (n=3) were prepared from a stock solution (1000 μg/mL) in methanol-water (9:1). Working solutions of 200, 100, 50, 25, 12.5, 6.25 and 3.125 μg/mL were prepared daily. The linearity of the method was determined ($r^2 > 0.99$) and the limit of detection (LOD) and limit of quantitation (LOQ) were calculated from the mean for the slope of the calibration curves, and the standard deviations (S.D.) of the intercepts for the calibration curves using a signal-to-noise
ratio (S/N) of 3.3 for the LOD, and 10 for the LOQ [25, 26]. The intraday precision was estimated using the areas under the curve at three concentrations (100, 25 and 6.25 µg/mL) in one day. Interday precision was analysed using those three concentrations on three different days. The precision was evaluated after 9 injections of salvinorin A (50 µg/mL) and the result was compared with the nominal value.

2.6 Statistical analysis

All statistical analyses were carried out using the SigmaStat 3.5 software.

3. Results and discussion

3.1 Validation of the HPLC method

The intraday precision was calculated by comparing the Coefficient of Variation (CV) obtained after three injections of salvinorin A in a single day for 100, 25 and 6.25 µg/mL, resulting in CVs of 4.75, 0.34 and 1.09 %, respectively. The interday precision was calculated for these concentrations, resulting in CVs of 2.88, 1.28 and 1.37 %, respectively. Additionally, a one-way ANOVA for the interday variability did not show statistical difference for the areas under the curve (p> 0.05 One way ANOVA). In addition to showing a CV lower than 5%, the one-way ANOVA confirmed the reproducibility of our method.
In this study, the precision of the method was calculated after nine injections of one concentration of salvinorin A (50 µg/mL), obtaining a quantity of salvinorin A detected 50 ± 0.88 µg/mL (mean ± S.D.). The variability of 1.76%, for the HPLC method can be considered acceptable since variability smaller than 3% is required for a method to be accepted as precise [26].

The linearity of the method (Table 1) was determined from the calibration curves on three different days and three replicates of each, using seven concentrations (200-3.125 µg/mL) of salvinorin A and obtaining a correlation coefficient ($R^2$) greater than 0.999. According to this result, the linearity of the method complies with the requirements for the validation of quantitative HPLC methods [26, 27]. In this study we determined the linearity of the method by determining the value of $R^2$. However, there are reports of the use of statistical analysis such as ANOVA for the determination of the linearity [28, 29]. In our experiments, salvinorin A showed a retention time of 23.09 ± 0.95 min.

The calculated LOD and LOQ for this method were 0.44 and 1.34 µg/mL, respectively. Although our method has higher LOD and LOQ values than those reported elsewhere for the analysis of salvinorin A [10, 27], it is still able to detect and quantify concentrations above 1340 ng/mL.

3.2 Salvinorin A content in commercial products of S. divinorum
The extraction process was carried out using a methodology previously established using a sonication time of 1 h [25].

Table 1. Calibration curves for the determination of linearity in the HPLC method.

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Area* (V x sec)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
<td>Day 3</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>3328.4 ± 51.50</td>
<td>3258.2 ± 36.45</td>
<td>3202.2 ± 25.02</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>1687.95 ± 80.33</td>
<td>1632.19 ± 22.76</td>
<td>1612.5 ± 41.00</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>858.23 ± 40.85</td>
<td>821.71 ± 29.23</td>
<td>807.84 ± 13.53</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>396.28 ± 1.33</td>
<td>394.65 ± 6.65</td>
<td>383.29 ± 7.245</td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td>194.83 ± 8.27</td>
<td>189.15 ± 8.34</td>
<td>191.17 ± 20.87</td>
<td></td>
</tr>
<tr>
<td>6.25</td>
<td>107.63 ± 1.20</td>
<td>102.19 ± 0.91</td>
<td>97.11 ± 2.26</td>
<td></td>
</tr>
<tr>
<td>3.125</td>
<td>49.52 ± 1.69</td>
<td>47.39 ± 2.75</td>
<td>48.98 ± 2.24</td>
<td></td>
</tr>
<tr>
<td>R²</td>
<td>0.9998</td>
<td>1.000</td>
<td>0.9999</td>
<td></td>
</tr>
</tbody>
</table>

*The mean value of area of injection (n = 3) for each concentration tested ± S.D.

The concentration of salvinorin A in the concentrated leaf extract samples ranged between 8.32 - 56.52 mg/g dry leaf. Table 2 provides a summary of the
concentrations of salvinorin A detected in each commercial product (mean ± S.D.). Although we observed a correlation between the strength of the products and the amount of salvinorin A detected ($R^2 = 0.941$ for brand a and $R^2 = 0.962$ for brand c), it is evident that these do not correspond to the amount expected for each strength. Additionally, there was no consistency between the detected amounts of salvinorin A, for a given strength, among the different brands used in this study. It is noticeable that none of the samples from brand c contained a higher concentration of salvinorin A than the 40X sample from brand a. In the preparation of commercial products of $S. divinorum$, the leaves are commonly used since previous studies have shown the highest concentration of salvinorin A in this part of the plant [30]. According to the EMCDDA (European Monitoring Centre for Drugs and Drug Addiction), based on a study by Jermain and Evans in 2009 [18], the strength 1X is equivalent to the amount of salvinorin A in dry leaves (0.408 mg/g); however, several studies report a high variability in this amount (0.89 – 3.7 mg/g) depending on the freshness of the leaves [31], the adulteration with products such as caffeine or vitamin E [32], the geographic origin and cultivation method of the plant material [27, 32, 33], or the use of other aerial parts of the plant in the production process [10]. All these factors have an effect on the content of salvinorin A per gram of product and adds ambiguity to the real strength of the commercial products.
Table 2. Salvinorin A detected in commercial products using HPLC analysis.

<table>
<thead>
<tr>
<th>Declared Strength of S. divinorum product</th>
<th>Declared [mg/g] of Salvinorin A on label</th>
<th>[mg/g] Salvinorin A measured by HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>5Xa</td>
<td>---</td>
<td>14.32 ± 0.90</td>
</tr>
<tr>
<td>10Xa</td>
<td>---</td>
<td>25.12 ± 1.72</td>
</tr>
<tr>
<td>15Xa</td>
<td>---</td>
<td>33.18 ± 3.11</td>
</tr>
<tr>
<td>20Xa</td>
<td>---</td>
<td>29.50 ± 1.62</td>
</tr>
<tr>
<td>40Xa</td>
<td>---</td>
<td>56.52 ± 3.77</td>
</tr>
<tr>
<td>5Xb</td>
<td>---</td>
<td>12.03 ± 0.78</td>
</tr>
<tr>
<td>5Xc</td>
<td>16</td>
<td>8.32 ± 0.65</td>
</tr>
<tr>
<td>10Xc</td>
<td>28</td>
<td>17.00 ± 0.80</td>
</tr>
<tr>
<td>20Xc</td>
<td>40</td>
<td>21.37 ± 1.56</td>
</tr>
<tr>
<td>40Xc</td>
<td>80</td>
<td>28.91 ± 1.32</td>
</tr>
<tr>
<td>70Xc</td>
<td>120</td>
<td>36.32 ± 1.65</td>
</tr>
<tr>
<td>100Xc</td>
<td>160</td>
<td>54.80 ± 2.72</td>
</tr>
</tbody>
</table>
Values are for the mean ± S.D. for each product. The designation ‘a, b, c’ corresponds to a different brand name.

A one way ANOVA analysis between the three 5X strength samples revealed a statistical significance \( F_{(2,24)} = 238.148, p<0.001 \) for salvinorin A content among the brands. For the 10X, 20X and 40X samples only two brands were available, hence, a Student-t test was carried out. The amount of salvinorin A present in the different brands showed statistical significance, 10X \( [t= (15.683, 16) p<0.001] \), 20X \( [t= (14.077, 16) p<0.001] \), 40X \( [t= (28.450, 16) p<0.001] \) (Fig.2). Additionally, the relationship between actual strength and concentration of salvinorin A claimed on the packaging was determined as shown in Table 3.

Our findings for all strengths of brand c showed that the samples have a concentration of salvinorin A lower (30.24 – 60.67%) than those claimed on the packaging labels. The variability between the measured concentrations of salvinorin A for different products has also been observed in two previous studies carried out in US and Japan. In the study of Wolowich et al., 2006, the concentration calculated and the declared varied between 1-16% [32]. Whereas, Tsujikawa et al 2009 report higher concentrations of salvinorin A that the concentration reported for all samples analysed [10]. Recently, Moreira et al., 2014 have also highlighted a lack of consistency between declared and calculated amounts of salvinorin A in *S. divinorum* products [34].
Fig. 2. Statistical test in samples of *S. divinorum*. One-way ANOVA was used for comparison of the 5X samples. Values are expressed as mean ± S.D. *p<0.01* vs 5Xc, **p<0.01** vs 5Xb. For the comparison of the 10X, 20X and 40X samples, a
Student t-test was used. Values are expressed as mean ± S.D., *p<0.001. The tests were carried out using SigmaStat 3.5.

Table 3. Potency-concentration detected in samples c.

<table>
<thead>
<tr>
<th>Strength</th>
<th>Relationship expected*</th>
<th>Relationship observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X – 10X</td>
<td>1.75</td>
<td>2.04</td>
</tr>
<tr>
<td>5X – 20X</td>
<td>2.5</td>
<td>2.56</td>
</tr>
<tr>
<td>5X – 40X</td>
<td>5</td>
<td>3.47</td>
</tr>
<tr>
<td>5X – 70X</td>
<td>7.5</td>
<td>4.36</td>
</tr>
<tr>
<td>5X – 100X</td>
<td>10</td>
<td>6.58</td>
</tr>
</tbody>
</table>

* The relationship expected was calculated from the concentration declared in the packaging box.

3.3 Potential Health Risk associated with S. divinorum consumption

Consumption of commercial products of S. divinorum as legal highs has become very popular among young adults worldwide [35] because of its legal status, easy availability, the absence of toxicological effects, and the positive description of the events that are reported in blogs and websites [36, 37]. Although salvinorin A is
reported to produce strong hallucinogenic effects at concentrations of 200-500 µg [7]; the behavioural and physiological effects experienced by consumers are determined by the amount of salvinorin A in the product being consumed, which, as we demonstrated in this study, shows a great variation across a random sample of commercial products available in Mexico [38, 39]. Based on the calculated concentrations of salvinorin A for the products evaluated in this study, we could assume that 24 to 60 mg of sample 5Xc will be enough to cause hallucinogenic effects. Moreover, a similar amount of the product 40Xc, far from producing the desired positive behavioural effects, could reach levels high enough to produce adverse effects that can increase the risk for accidents [39, 40]. It is important to note that S. divinorum products with 100X strength were readily available at internet shops.

Additionally, the consumption of S. divinorum with other substances such as alcohol and other drugs has been reported as a common habit [41]. However, data recording the long-term effects or interactions due to the combination of legal and illegal recreational drugs with S. divinorum are still scarce [42]. It has been reported that salvinorin A modulates the action of cocaine through the interaction with dopamine receptors D₁ [43]. It has also been reported that chronic consumption of S. divinorum is linked to memory and cognitive development damage [44]. The consumption of this legal high has been associated with short-term risks such as the development and triggering of psychotic episodes in patients predisposed to schizophrenia [44]. Finally, although S. divinorum has only shown a
low toxicity in rodents, there is a need for information regarding its long term effects in humans.

4. Conclusion

We validated an HPLC method to quantify the amount of salvinorin A in S. divinorum products sold in Mexico. Similarly to reports for S. divinorum products sold in Japan, US and Portugal, our study found no consistency in the amount of salvinorin A content when different brands and strengths of S. divinorum products were compared. Additionally, for those samples showing on the packaging box the amount of salvinorin A content, these did not match the amount calculated. Finally, it is important to have a full understanding of the long-term neuropsychiatric effects of S. divinorum in order to prevent and minimise potential public health risks.

5. References


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Highlights

- Commercial products of *Salvia divinorum* available in Mexico were purchased
- An HPLC method for the quantification of salvinorin A was validated
- The amount of salvinorin A in the purchased products was determined
- The strength of *S. divinorum* products is relative to the brand